

## THE CELLULAR STRUCTURE OF THE LEYDIG ORGAN IN THE SHARK, *ETMOPTERUS SPINAX* (L.)

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### ABSTRACT

In *Etmopterus spinax*, a small deep water shark, the predominating lymphomyeloid tissue is the so called Leydig organ. This consists of bone marrow-like tissue situated between the muscularis and the mucosa of the esophagus. Examination by light and electron microscopy shows that the Leydig organ produces large numbers of granulocytes and lymphocytes. Two main types of granulocytes occur, tentatively called heterophilic and eosinophilic. The heterophilic cells may be subdivided into three types which differ in the ultrastructure of the granules. Cells structurally resembling mammalian plasma cells are common. The presence of these cells indicates that the tissue is part of the shark immune system.

### INTRODUCTION

In 1685 the Danish anatomist, Nicolaus Steno, noticed a gland-like structure in the esophagus of a ray, *Raja*. Probably the same structure was later found in several elasmobranchs, and Leydig (1857) interpreted it as a lymph node. Later authors ascribe it both lymphoid and myeloid activities. Therefore the term lymphomyeloid may be appropriate to describe this kind of tissue. The esophageal lymphomyeloid tissue of elasmobranchs is often called the Leydig organ (organ of Leydig). In some elasmobranchs the Leydig organ is remarkably large. Bolton (1927) found it to weigh about 1.6 kg in one 1.8 m long cow shark (*Hexanchus corinus*), and 1.2 kg was noted in a 2.9 m Greenland shark (*Somniosus microcephalus*) by one of the authors (R.F.).

Certain elasmobranchs lack the Leydig organ but instead possess lymphomyeloid tissue associated with the gonads, the epigonal organ. Most species of elasmobranchs possess both Leydig and epigonal organs.

In recent years a growing number of investigators have become interested in the evolution of the immune system. It has been clear that elasmobranchs show strong immune responses (see review by Marcharlonis, 1977). In connection with the findings of immunological activity it is important to reinvestigate the various lymphomyeloid structures which form the structural basis of immune processes.

Histological studies of the Leydig organ have been done by Pilliet (1890), Drzewina (1905, 1910), Petersen (1908), Kulchitzkii (1911), Maximov (1923), Kanasada (1956), Fey (1965) and Fänge (1968). The ultrastructure of the Leydig organs of the rays *Raja clavata* and *Torpedo marmorata* has been investigated by Zapata (1981) and of the dogfish, *Scyliorhinus canicula* (by R.F. and A.P., Marine Biol. Assoc. UK, 1981, in preparation).

The shark, *Etmopterus spinax*, belongs to those species of elasmobranchs which have a well developed Leydig organ but lack the epigonal lymphomyeloid structure. The present study describes the ultrastructure of cells from the Leydig organ of

*Etmopterus spinax*. In addition we discuss histological data obtained from a material larger than that used earlier (Fänge, 1968). By using both light microscopical and ultrastructural criteria most cells can be equalized with those from mammalian lymphomyeloid systems.

## MATERIALS AND METHODS

The animal material consisted of about 50 specimens of the blue velvet, *Etmopterus spinax*, a small deep water shark occurring in the North Atlantic. The body weights varied between 12 g to 600 g (adult females). The fishes were caught in the Scagerack Sea from depth of 200–400 m by trawling. (A few specimens were infested by the cirripedian parasite, *Anelasma squalicola*). Fresh samples of tissues were prefixed on board the ship.

For histology pieces of tissues were fixed in 10 per cent neutral formalin and examined by routine methods after staining with eosin-haematoxylin or Giemsa. Imprints (touch preparations) were air dried 1 hour, fixed for 15 min in absolute methanol and stained with May-Grünwald-Giemsa (MGG).

For electron microscopy small cubes of tissue, about 1 mm<sup>3</sup>, were fixed in 3 per cent glutaraldehyde, pH 7.3 for 1–3 days. This solution was made up either of 0.2 M cacodylate buffer or in a mixture of one third veronal acetate buffer and two thirds sea water. To this latter solution sucrose was added to a final concentration of 5 per cent as was a trace of CaCl<sub>2</sub> (cf. Bell *et al.*, 1969). All samples were postfixed in 1 per cent OsO<sub>4</sub> dissolved in sea water. After dehydration in a series of ethanol the cubes were embedded in Epon 812 and sectioned on an LKB ultratome. The sections were contrasted with uranyl acetate followed by lead citrate and examined in a Hitachi HS-8 electron microscope at magnifications varying from 1,900× to 47,000×. The study is based on more than 500 electron micrographs. Thick sections (about 1 μm) adjacent to the thin sections studied in EM were stained with toluidine blue for light microscopy.

## RESULTS

### *Light microscopy*

The Leydig organ constitutes two whitish masses in the dorsal and ventral parts of the esophagus (Fig. 1). The tissue consists of enormous numbers of mature and immature leucocytes within a connective tissue stroma. Lobes formed by tightly packed cells are separated by irregular venous spaces. The lobes are penetrated by small arteries. Histologically the tissue resembles hemopoietic bone marrow of terrestrial vertebrates, although it does not form red cells and contains no fat tissue. As previously found (Fänge, 1968) the predominating cells are two types of granulocytes and non-granulated cells with basophilic cytoplasm. The heterophilic granulocytes are more frequent than the eosinophilic ones. The heterophilic granulocytes contain fine rod-shaped weakly eosinophilic granules. In imprints these cells measure 17–25 μm (width) by 20–28 μm (length), whereas the same cells appear about half that size in histological sections (9–12 μm × 10–15 μm). The eosinophilic granulocytes are of similar size but usually more round. They contain rather large (1–2 μm) intensely eosinophilic granules (Fig. 2A). The majority of the granulocytes appear to be in the myelocyte stage and often show mitoses.

Types of non-granulated cells identified in MGG-stained imprints are lymphocytes of various sizes, spindle-shaped cells (thrombocytes), blast cells (probably mainly granuloblasts), plasma cells and monocytes. However, the separation by

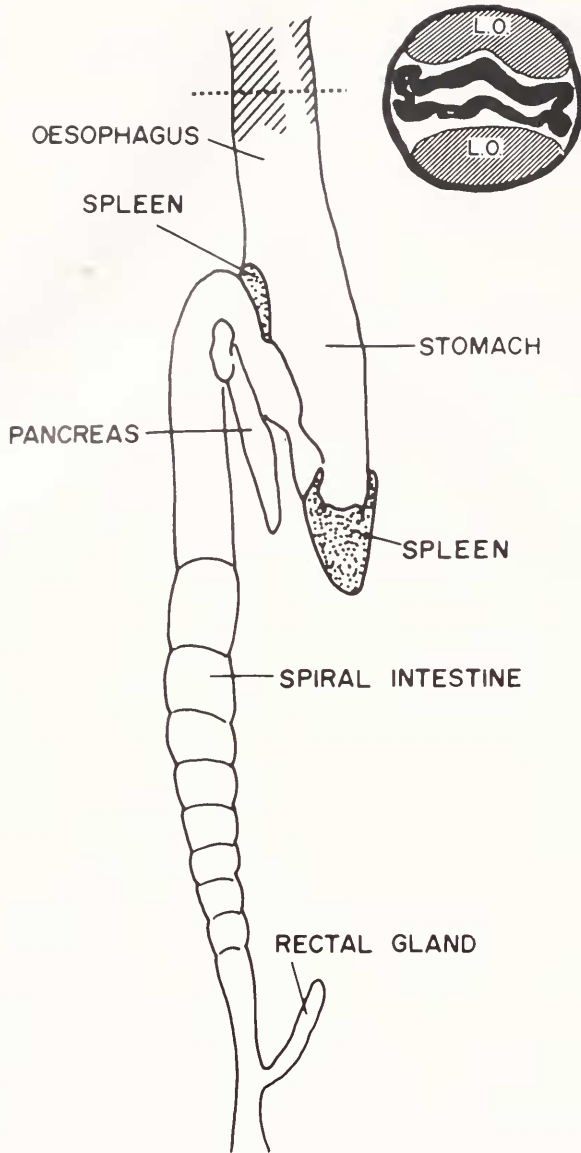


FIGURE 1. Ventral view of the digestive tract showing the localization of the Leydig organ (shaded). To the right an enlarged and transversal section through the esophagus (at the broken line) showing dorsal and ventral portions of the Leydig organ (L.O.).

light microscopy of blast-type cells and plasma cells is uncertain, since both cell types possess a large nucleus and an intensely basophilic cytoplasm. An eccentric position of the nucleus and a clear zone next to the nucleus are features which may be characteristic of plasma cells. It is also difficult to distinguish between small lymphocytes and spindle cells (thrombocytes).

The leucocytes of the Leydig organ often aggregate into diffuse groups or follicles (Fig. 2B). This especially concerns blast cells, eosinophilic granulocytes and

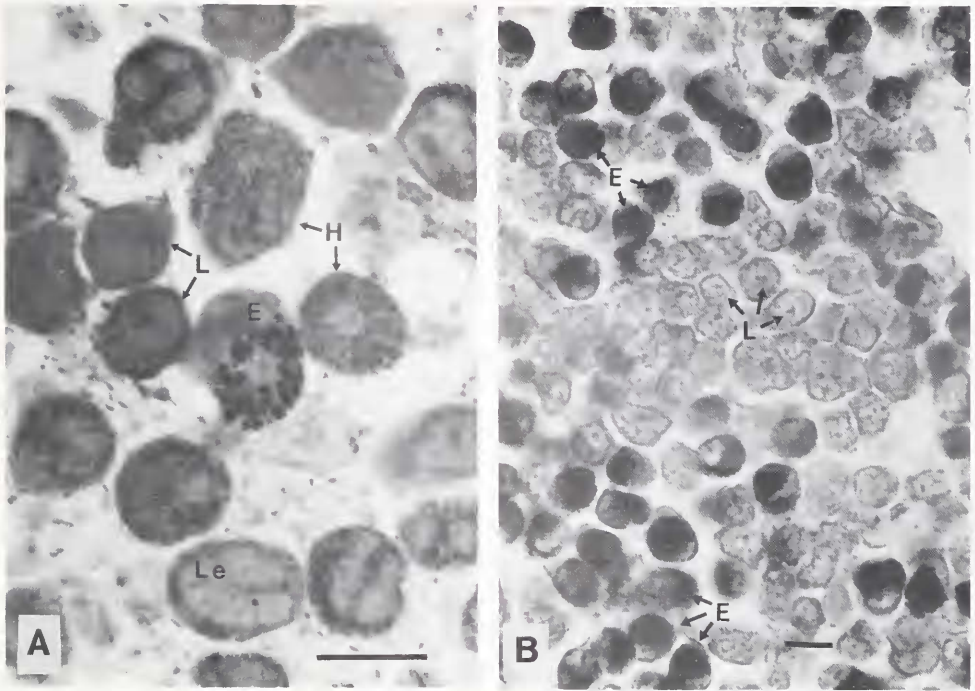


FIGURE 2. Light microscopy of the Leydig organ of *Etmopterus*.

(A): Epon-embedded section, about  $1\ \mu\text{m}$  thick and stained with toluidine blue. In the middle an eosinophilic granulocyte (E). Lymphocytes (L) and heterophilic granulocytes (H) are seen around the eosinophilic cell. At the bottom a typical leptomeric cell (Le) with invaginated nucleus. Oil immersion. Bar =  $10\ \mu\text{m}$ .

(B): Azan-stained paraffin section. In the middle of the figure a group of non-granulated cells, mainly lymphocytes. Above and below these cells there are eosinophilic granulocytes (dark cells). Bar =  $10\ \mu\text{m}$ .

small lymphocytes. In Giemsa-stained histological sections the tissue appears as a mosaic of red-stained granulocytes and bluish non-granulated cells.

### *Electron microscopy*

Most cells of the Leydig organ show ultrastructural characteristics resembling those of blood cells from various vertebrates and even invertebrates (Bessis, 1973; Mattisson and Fänge, 1977). The same types of leucocytes as identified by light microscopy are also found by the electron microscope, but the more structural details obtained are important for a further identification of the leucocytes.

*Eosinophilic granulocytes.* On electron micrographs the granules measure up to  $1.5\ \mu\text{m}$  in diameter and have a spheric form (Fig. 4). In contrast to generally described eosinophils of higher vertebrates those of the *Etmopterus* Leydig organ contain granules without a crystalline core. The periphery of the granules often has an electron lucent frame. Compared with the heterophilic cells described below the eosinophils are less densely packed with granules. Surrounding the granules a rough endoplasmic reticulum is often found (Fig. 4). The eosinophils seem to make up only about 5% of the total number of granulocytes.



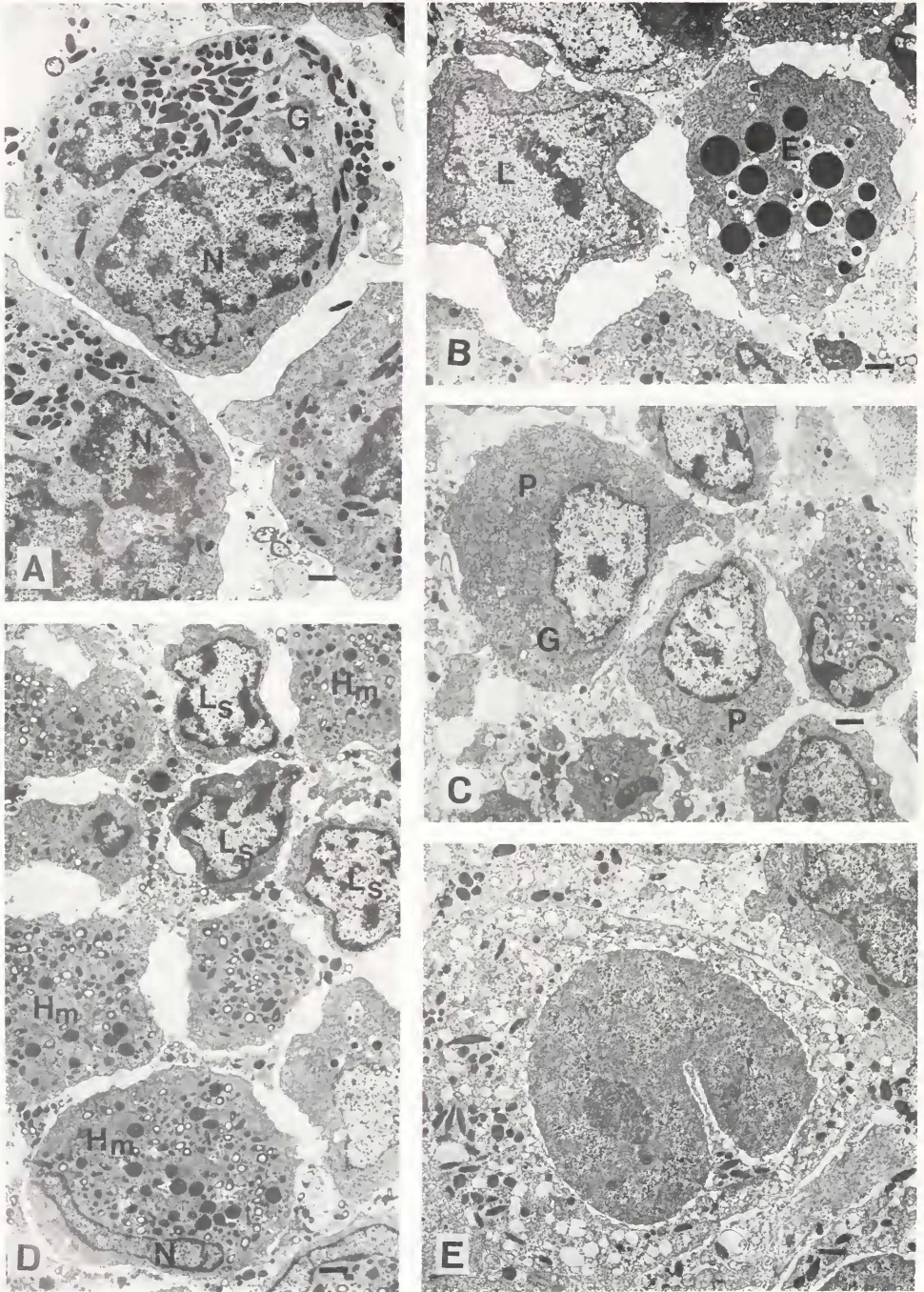


FIGURE 3. Electron microscopy of different cell types from the Leydig organ. Bar = 1  $\mu$ m.

(A): The most common heterophilic granulocytes, designated type A. Ovoid and rod-shaped granules, a few of them in the intercellular space. Lobed nuclei (N). Golgi apparatus (G). Fixation for 1–3 days in 3% glutaraldehyde dissolved in 0.2 M Na-cacodylate buffer. Postfixation for 1 h in 1% OsO<sub>4</sub> in sea water. The fixation media were adjusted to neutral pH.

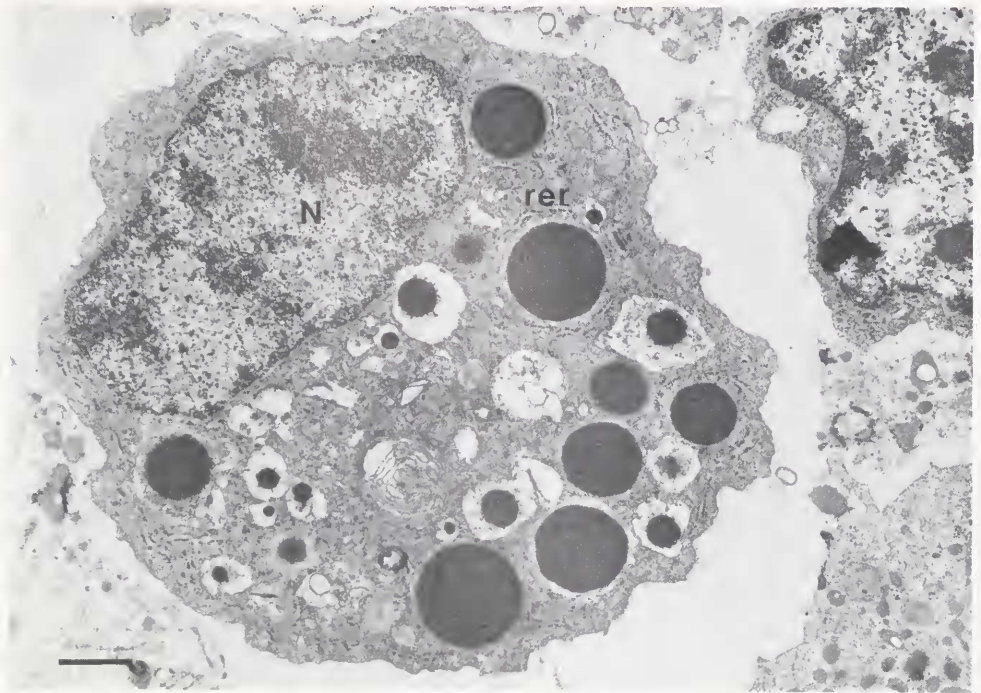


FIGURE 4. Eosinophilic granulocyte. Nucleus (N) at the periphery of the cell. The cytoplasm contains a few spheric and homogeneously dense granules. Each of the smaller dense spheres is surrounded by a "clear area." Between the granules is a well developed rough endoplasmic reticulum (rer). Fixation as in Figure 2B. Bar = 1  $\mu$ m.

*Heterophilic granulocytes.* Ultrastructurally the granules are ovoid about 0.5  $\mu$ m by 1  $\mu$ m (Figs. 3 and 5). Solitary granules, however, may be rod-shaped with a length 4–6 times their width. As seen in Figure 5 the granules contain filamentous longitudinal internal structures. The heterophils often have a highly lobed nucleus and close to this there are often annulate lamellae. Next to the nucleus there is a well developed Golgi apparatus and numerous vesicular structures, which probably are primary lysosomes. However, the heterophilic granulocytes vary much in

(B): Large lymphocyte or blast cell (L) and eosinophilic granulocyte (E). The latter has spheric and homogeneously dense granules, some of them surrounded by an "empty" region. Fixation as in Figure 3A but glutaraldehyde was dissolved in a mixture of veronal acetate and sea water (1:2). This solution was supplied with sucrose to a final concentration of 5% and a trace of  $\text{CaCl}_2$ . Adjustment to neutral pH with NaOH.

(C): Two plasma cells (P) dominate the figure. Besides the typical ribosome-coated endoplasmic reticulum the cytoplasm of the left cell has a few dense granules close to the Golgi apparatus. Both cells show "empty" areas within the nuclei. Fixation as in Figure 2B.

(D): Small lymphocytes ( $L_s$ ) and modified heterophilic granulocytes ( $H_m$ ). The lymphocyte nuclei have areas with strongly electron dense chromatin. The modified granulocytes, designated type B, show characteristic slender "saucer-shaped" nuclei (N) and granules with central electron lucent regions. Fixation as in Figure 2B.

(E): Another type of modified heterophilic granulocyte, designated C. This type is characterized by a great number of electron lucent areas (degranulated areas?) mingled with the common type of heterophilic granule and it has a leptochromatic nucleus pierced by a string of cytoplasm. Fixation as in Figure 2A.





FIGURE 5. Portion of a heterophilic granulocyte (type A). The cytoplasm is well supplied with electron lucent small vesicles and granules of the common heterophilic type, some of them with a filamentous interior (arrows). Nucleus (N) with a granule-like body (arrow head), mitochondria (m), multivesicular body (mb) and Golgi apparatus (G). Fixation as in Figure 2A. Bar = 1  $\mu$ m.

their ultrastructure. Based on differences in the structure of the granules we tentatively distinguish three sub-types of heterophilic granulocytes, here designated types A, B and C.

Type A. (Figs. 3A, 5). The granules of this type of heterophilic cell are homogeneously electron dense. Type A cells constitute about two thirds of the total number of heterophils. However, different types of heterophils occupy special areas within the Leydig organ and therefore this estimation of the number of type A cells is only an approximation.

Type B. (Fig. 3D). The cells are smaller than those of A-type. The smaller granules have a central vesicular structure with electron lucent interior. The B-cells have a very characteristic saucer-shaped nucleus.

Type C. (Fig. 3E). These cells are 6–10  $\mu$ m in diameter, have a very loose cytoplasm and show degenerate features. The cytoplasm contains membrane-bound vesicles with a size and shape agreeing with the granules of the other heterophils. Some of the cells show a highly reduced electron density all over the cells. Type B- and type C-cells are found in about the same number. In both B- and C-cells there often occur granules of the common heterophilic type (type A-cells).

With electron microscopy we often found free intact granules in interstitial regions (Fig. 3A). The presence of free leucocytic granules is also noticed in almost all imprint preparations and has previously been reported by Fey (1966) from the dogfish (*Scyliorhinus canicula*). Besides these free granules a special type of spheric body occurs between the cells as well as inside cells of monocyte type. These granules may be phagocytic inclusions or they may be formed inside the cells. They are

up to 2  $\mu\text{m}$  in diameter and have a moderate density suggestive of lipids or lipoproteins (Fig. 6). When occurring in the cytoplasm the bodies are surrounded by a circle of dense irregular grains.

Small lymphocytes in the electron microscope measure 4–6  $\mu\text{m}$  in diameter and have a large nucleo-cytoplasmic ratio (Fig. 3D). The nucleus has a dense, mainly peripheral heterochromatin and the cytoplasm has a high content of free ribosomes. Another category of lymphocyte-like cells measures 8–12  $\mu\text{m}$  in diameter. Their more extensive cytoplasm is richly supplied with free ribosomes as well as with polysomes. The nucleus has a well developed nucleolus. These cells may be large lymphocytes or blast cells. As granulocytes are predominant, many blast cells probably are granuloblasts (Fig. 3B).

Among the lymphocyte-like cells there are some which are difficult to identify with known cell types. Some of them have granules similar to those of heterophilic granulocytes. Others have a leptochromatic nucleus pierced by a cytoplasmic invagination. The latter may be "pale transition cells" (Yoffey, 1980).

Cells containing vacuoles of varying sizes, microtubules, filaments and a special type of granules may be considered as monocytes (Fig. 6). Their specific granules are about 0.2  $\mu\text{m}$  in diameter and supplied with a thin halo. This type of granule is often, e.g. by Bessis (1973), described as azurophilic granules. In addition to the azurophilic granules one also observes granules with lipid density.

The plasma cells, or plasma cell-like cells, measure up to 15  $\mu\text{m}$  in diameter. The cytoplasm shows an ultrastructure similar to that of mammalian plasma cells (Figs. 3C, 7). A well developed Golgi apparatus and a vast system of rough endoplasmic reticulum reflects a high level of protein synthesis. The reticulum forms sac-like structures richly supplied with ribosomes (Inset Fig. 7). Among the endoplasmic membranes there are often solitary granules appearing like heterophilic granules. The nuclei show some characteristics deviating from those of mammalian plasma cells. They are generally central, comparatively large and with irregular outlines, and they do not show any cartwheel-like arrangement of their heterochromatin, characteristic of mammalian plasma cells. In the assumed plasma cells of *Etmopterus* the heterochromatin is concentrated in a nucleolus-like structure and in a thin frame bordering the nuclear membrane. In some cases the nucleus has a region deviating by a lack of heterochromatin but having a homogeneous appearance. This region may be electron transparent (Fig. 3C) or have a density agreeing with that of the cytoplasmic sacs (Fig. 7).

#### DISCUSSION

The Leydig organ belongs to the same category of tissues as the epigonal organs of elasmobranchs, the lymphomyeloid tissues of the orbit and the roof of the mouth in holocephalians, and the meningeal lymphomyeloid tissues of chondrosteans and holosteans. These are bone marrow-like tissues which are mainly granulopoietic but also produce lymphocytes.

In *Etmopterus* the Leydig organ is well developed and like the spleen weighs about 0.5% of the body weight (Fänge, 1977). In its microscopical and electron microscopical structure it closely resembles the epigonal organ occurring in many elasmobranchs (Fänge and Mattisson, 1981).

The electron microscope reveals the presence of numerous cells with several characteristics of plasma cells. The cytoplasm of these cells is a structural copy of that from mammalian plasma cells. The characteristic cisternae of endoplasmic



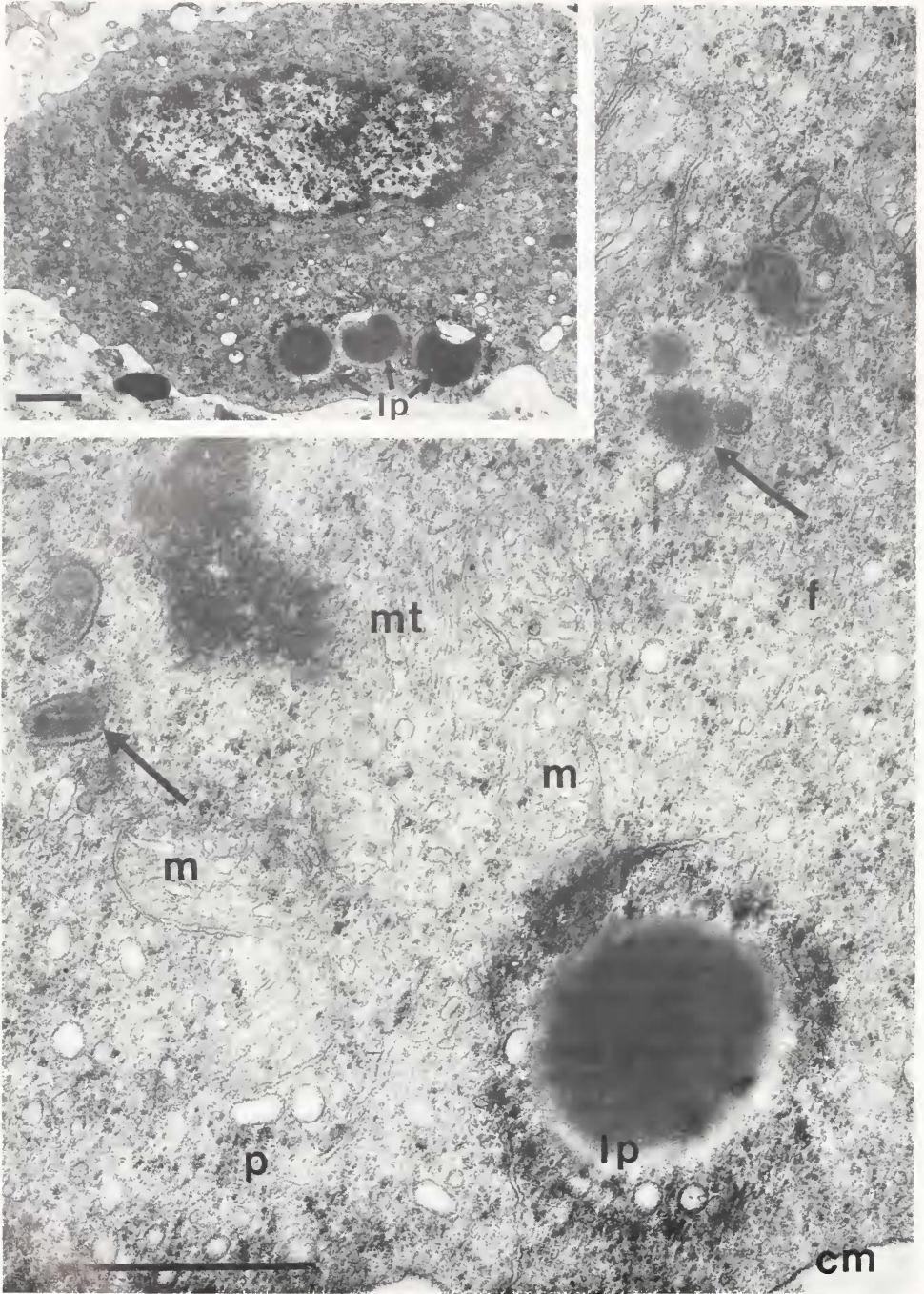


FIGURE 6. Portion of a monocyte-like cell. The cytoplasm contains halo-supplied granules appearing like azurophilic ones (arrows), mitochondria (m), microtubules (mt), polyribosomes (p) and filaments (f). Close to the cell membrane (cm) a large "lipoprotein" granule (lp) surrounded by dense grains. Inset: "Lipoprotein" granules in another monocyte-like cell. Fixation as in Figure 2A. Bars = 1  $\mu$ m.

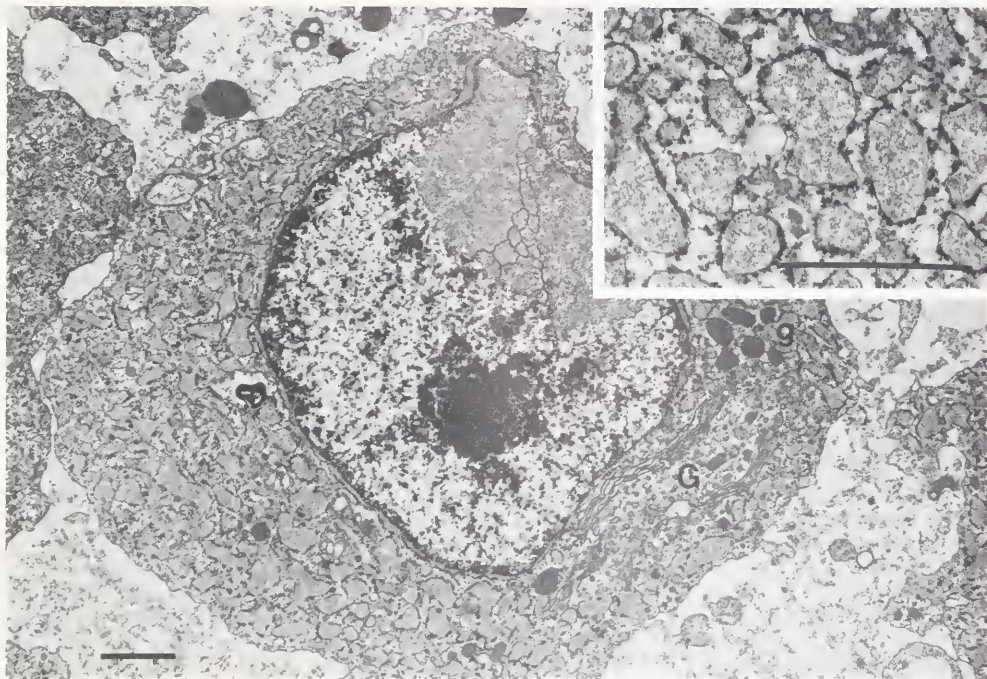


FIGURE 7. Plasma cell. The cytoplasm is filled by rough endoplasmic reticulum in the shape of vesicles containing a moderately dense substance. Close to the Golgi apparatus (G) are some lysosome-like granules (g). In the large nucleus there is a homogeneous moderately dense area divided into smaller portions by delicate membranes. The heterochromatin is centrally localized and forms a thin peripheral frame. Inset: Portion of the cytoplasm showing the ribosomes on the outer part of the vesicular membranes and the moderately dense interior of the vesicles. Fixation as in Figure 2B. Bars = 1  $\mu$ m.

reticulum are well supplied with ribosomes indicating that the main function of the cells is the synthesis of proteins. The identification of these proteins as immunoglobulins remains to be done. By light microscopy Good *et al.* (1966) identified plasma cells in various lymphomyeloid organs from sharks and rays. Fänge and Mattisson (1981) found plasma cells in the spleen and also in the epigonal organ of the nurse shark, *Ginglymostoma cirratum*. Ellis (1977), without exactly identifying the cell type, observed cells carrying immunoglobulins in the Leydig organ of the ray, *Raja naevus*. Several studies have demonstrated efficient production of immunoglobulins as a response to antigenic stimulation in elasmobranchs (Litman *et al.*, 1976; Marcharlonis, 1977). The presence of great numbers of plasma cell-like cells in the Leydig organ of *Etmopterus* leads us to conclude that this tissue probably has important immune functions.

The electron microscopic studies confirm earlier findings of two types of granulocytes in *Etmopterus* and have contributed further information on the disparity of the granules. In accordance with Fey (1966) we use the term heterophilic for the predominant type of granulocyte. These finely granulated cells may resemble the neutrophils of higher vertebrates, but their granules are eosinophilic rather than neutrophilic. The other main type of granulocyte, here termed eosinophilic, contains large spheric granules which are strongly eosinophilic by light microscopy. The



granules differ from those of mammalian eosinophils by their homogeneous electron density lacking crystalline structures. A homogeneous interior of eosinophilic granules was reported by Clawson *et al.* (1966) in the paddlefish (*Polyodon spatula*), a chondrosteian fish, by Kélenyi and Németh (1969) in certain primitive fish, and by Morrow and Pulsford (1980) in the dogfish (*Scyliorhinus canicula*).

In addition to the two main types of granulocytes the electron microscope shows cells which appear to be modifications of the heterophilic granulocyte. The discrepancy mainly concerns the granules. Some deviating cells show granules with central translucent vesicles like those described by Kélenyi and Németh from reptiles and amphibia (1969). In other cells most granules appear completely translucent. This structural variation may represent developmental or functional stages. Morrow and Pulsford (1980) likewise report the occurrence of different types of heterophilic granulocytes in the dogfish (*Scyliorhinus canicula*).

The Leydig organ contains numerous large lymphocyte-like cells with distinct nucleoli and abundant cytoplasmic ribosomes. The cellular structure indicates that the majority of these cells may be granuloblasts. As to the small lymphocytes in our electron microscopic study, we have difficulty in distinguishing them from thrombocytes (spindle cells). This difficulty has also been reported for other species of fish by Ellis (1976) and Ferguson (1976).

Several cells show signs of endocytotic activity. Some of these often contain spheric bodies of lipid or lipoprotein density surrounded by electron dense granules (explosions caused by electron bombardment?). These cells also have halo-supplied granules and appear like monocytes or macrophages. However, as asserted by Ellis (1977), the occurrence and designation of monocytes in fishes is confused and contradictory.

Granulocytes and lymphocytes often have pale nuclei pierced by invaginated cytoplasm (Fig. 2E). Yoffey (1980) describes such leptochromatic cells in mammals as "transition cells." The leptochromacia indicates a high activity. In cultures of bone marrow such cells show marked streaming of nuclear material (Rosse, 1971). As to the granulocytes of *Etmopterus* the pale cells are most common among the B- and C-types with their more or less electron lucent granules. These cells often give the impression of being degenerate. The degenerate appearance of leucocytic granules may be due to release of substances from the granules during certain conditions. The substances released could be hydrolytic enzymes, as leucocytic granules from many animals are known to contain a wide array of such enzymes. The Leydig organ of *Etmopterus* is remarkably rich in lysozyme with a high chitinolytic activity (Lundblad *et al.*, 1979; Fänge *et al.*, 1980). *Etmopterus* very commonly are infested by a crustacean parasite, *Anelasma squalicola*. The processes of the parasite, which penetrate deep between skeletal muscle bundles of the shark, are provided with chitinous membranes. Perhaps the chitinolytic enzyme of the Leydig organ, undoubtedly of leucocytic origin, is part of a defense against parasites. However, so far we have not been able to correlate our structural findings such as observations of granulolysis in the leucocytes with the presence of the parasite.

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